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September 2, 1993

Commander Peter Kent, MD
Office of Naval Research
Combat Casualty Care Research Area
Naval Medical Research & Development Command
Naval Medical Command, National Capitol Region
Code 405
Bethesda, MD 20814-5044

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Subject: Periodic Administrative Report for Award N00014-90-J1797
Liquid Collagen Wound Coverings

Dear Commander Kent:

Attached is a brief summary of research progress since my last report of January 14, 1993. I apologize for its lateness.

Sincerely,

J. Peter Bentley, PhD
Professor and Interim Chairman
Biochemistry and Molecular Biology

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cc: Administrative Grants Officer
Director, Naval Research Laboratory
Defense Technical Information Center
Office of Chief Of Naval Operations
Bureau of Medicine and Surgery

Liquid Collagen Wound Coverings
Award Number N00014-90-J1797
Periodic Report
Summer, 1993

Freeze Dried Collagen Preparations

We have not pursued any further work with Oregon Freeze Dry, Inc. so the status is the same as that of our January 14, 1993 report.

Vehicle for Growth Factors

The studies of incorporation of fibroblast growth factor into slow release collagen based delivery vehicles was completed and was presented at a meeting of the Wound Healing Society held during the Keystone Symposia at Breckenridge, Colorado, March 28 through April 4. The material was presented as a poster which was well received and generated much comment. The data presented is attached.

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EXPERIMENTAL RESULTS WITH FGF (SCIOS) INCORPORATED INTO SLOW RELEASE COLLAGEN-BASED DELIVERY VEHICLES

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One of the major difficulties with the application of growth factors to accelerate wound healing is that to date there is no way to deliver the growth factor(s) constantly over a prolonged time. This would, we propose, greatly enhance the effect of the growth factor. We have developed a DOPA crosslinked type I collagen material (Gade, J. et al, 1991) and an iodine gelled type I collagen wound dressing for such purposes (U.S. Patents 5,128,136 and 5,081,106, enclosed). There are several techniques by which we are attempting to determine the feasibility of using these collagen-based materials as a delivery vehicle for growth factors, both in vitro and in vivo. These natural delivery vehicles have many advantages, including rapid gelling, moldability, good tensile strength no immunoreactivity (the collagen has been isolated by pepsin digestion), and their potential as slow-release vehicles. This preliminary study has concentrated on fibroblast growth factor FGF, as a model growth factor since it has been studied extensively and has been shown to enhance wound healing when applied topically in a liquid vehicle (unpublished).

EXPERIMENTAL RESULTS.

1. In Vitro determination of the rate of release of FGF from collagen matrices. These experiments were designed to determine the rate of release of FGF from DOPA and iodine gelled collagen matrices.

Procedure. In brief, FGF (SCIOS) with a small amount of ^{125}I FGF (Amersham) were added to the standard DOPA or iodine collagen mixes and gelling was allowed to occur (24 hr. for the DOPA collagen, 20 minutes for the iodine collagen). Gels were formed in 1 cc syringes: after gelling, the gels were removed from the syringe and cut into 0.2 ml sections. These gels, containing a known amount of ^{125}I FGF, were placed into 5 cc isotonic saline (PBS), and aliquots were removed at various time points for counting.

Results. As can be seen from fig. 1, about 20% of the growth factor was released from the gel over the first 30 minutes, whereas 80% of the FGF remained in the gel for at least one week. The same results were obtained with iodine gelled collagen. This indicates that the collagen matrices which we are currently investigating do act as a vehicle in which growth factor can be maintained for prolonged periods of time.

2. Assay of the activity of FGF incorporated into collagen matrices in vitro. In these experiments, we are attempting to answer two questions: first, is the FGF still active after being incorporated into the collagen matrix; second, will cells be stimulated to

migrate into the matrix (since 80% of the incorporated FGF remains in the matrix, it was thought this might act as an attractant for cells).

Procedure. Briefly, Human foreskin fibroblasts (HFF5 from Gary Shipley) were plated in 24-well plates at 10,000 cells/well in Fibroblast Basal Medium (FBM, Clonetics Inc.) with 5 μg/mL insulin. After two days, aliquots of growth factor in isotonic saline or pre-formed DOPA or iodine crosslinked collagen discs with and without varying amounts of FGF were added to the wells. After another 20 hr., any discs were removed for histology and cells were labeled for four hours with ^{3}H-dT to measure cell growth response. The discs were fixed in 10% formalin, fixed and stained to evaluate any cellular infiltration by light microscopy.

Results. As can be seen in figure 2, the cells respond in a dose-dependent manner to direct additions of FGF to the media. Growth response to FGF in iodine collagen discs, shown in figure 3, was not significant, even up to 5 ng/disc (in a 1mL culture). When the fixed and stained discs were examined by light microscopy, there were no cells apparent either at the periphery or within the disc. It should be noted that the cells deteriorated when presented with DOPA crosslinked collagen discs, and the addition of DOPA alone at comparable concentrations also caused the cells to deteriorate (by phase microscopy and by the fact that the cells incorporated only about 30% the amount of 3H-dT as control cultures). On the other hand, the cells incubated with iodine gelled discs appeared similar to and incorporated the same amount of 3H-dT as controls without a disc.

Comment. It may be that either the cells were not exposed long enough to the disc, or that a more appropriate method of presenting the material to the cells would be by coating the bottom of the culture dishes with our test materials. We therefore feel that further in vitro studies should be performed.

3. Rabbit ear cartilage replacement experiment. We are addressing the question of whether DOPA crosslinked collage will serve as a slow release vehicle for FGF in the rabbit ear cartilage model [FGF is known to stimulate chondrocyte growth (Cuevas,P. et al, 1988)]. Because DOPA crosslinked collagen has good mechanical properties and can be molded to any desired shape, it was proposed that this material might be a good replacement for cartilage (for example, in the ear or nose) and, with the appropriate growth factors in this case FGF, might stimulate ingrowth of cartilage into and ultimate replacement of the matrix with host cartilage elements.

Procedure. Very briefly, White New Zealand rabbits were anesthetized, and prepared for surgery. A slit was made through the skin of the inside of the ear, and the skin was separated from the cartilage using blunt scissors. A punch (5mM) was made through the cartilage - two on each ear - and the cartilage plug removed. The cartilage plug was replaced with either nothing (control), cartilage from the same plug (should close most rapidly), or a DOPA crosslinked collagen disc containing either 0, 0.25, 1, or 2.5 μg FGF. The wound was closed with sutures, and the rabbit allowed to recover. After 1,4,7, or 14 days the animals were sacrificed and the wound areas fixed in 10% formalin. Tissues were then processed for histology. Slides were examined and evaluated for cellular infiltrate and any response of the cartilage to the implant.

Results. Slides are still in the process of completion. Preliminary results, however, indicate a technical problem in that a portion of the implants appear to slide out of the hole, or wound area. It is apparent that a 5 mM cartilage plug wound like the one used in this method will take perhaps 8 weeks or longer to heal. When controls are compared to FGF containing discs, it is quite obvious (see figures 4 and 5) that wherever the cartilage is adjacent to a collagen disc containing FGF, the perichondrium is responds strongly by 14 days (there is no such response in controls). This is clearly a good indication that this methodology does permit the use of DOPA crosslinked collagen as a delivery vehicle for FGF. This also demonstrates that the FGF is active and capable of eliciting a response once incorporated into DOPA crosslinked collagen.

DISCUSSION

We have shown in these studies that DOPA and iodine gelled type 1 collagen acts as a slow release vehicle for FGF. It should also be noted that the rate and amount of release of FGF from these vehicles was the same when 50 mg/mL of heparin (SIGMA) was also incorporated into the discs. Although some of the rabbit ear discs also had heparin, it is not entirely clear whether the addition of heparin to these vehicles has any advantage in terms of added stability or better delivery as a direct comparison has not yet been made.

Secondly, we have shown that when FGF is incorporated into our collagen delivery vehicles, at least in the method used, human fibroblasts did not respond to the growth factor. We do not have a good explanation for these results at this time. We plan to test these materials by coating the tissue culture disc with our collagen vehicle, with or without FGF, and plate the cells on top of the vehicle to determine if they respond to the material presented in this way.

Third, we have shown that when FGF is incorporated into DOPA crosslinked collagen and tested in the rabbit ear cartilage replacement model, that there is an enhanced response of the host cartilage to a disc containing FGF as compared to controls. In another study where we tested control and PDGF-containing DOPA collagen discs in a rat dorsal implant assay, the PDGF was clearly active in stimulating cellular infiltration in great excess of controls (data not shown here). These results clearly indicate that the materials do have activity in vitro.

REFERENCES

Cuevas, R., Burgos, J. and Baird, A. 1988. Bioch. Bioph. Res. Comm. 156: 611-618

Gade, J.N., Fellman, J.H., and Bentley, J.P. 1991. J. Biom. Mat. Res. 25: 799-811

SUMMARY OF IN VIVO STUDIES WITH COLLAGEN-BASED GEL MATRICES

1. Pig Skin Experiment. In this experiment, either DOPA collagen or Iodine-collagen or a mixture of both was used as a pourable wound dressing. The animal model system was to make 2 cm full thickness punch wounds in the back of pigs. Fresh collagen mixtures were immediately poured into the wound site, and the wounds were covered with Opsite 15 min. later. Animals were observed daily, and were sacrificed and tissues processed for histologic examination on days 12 and 14. The results of this preliminary experiment showed that the collagen matrices were not detrimental to the wound. In fact, there was slightly better coverage with epithelium in the iodine-collagen treated wounds, and even greater epithelial coverage with the iodine and DOPA collagen mixture. It must be noted that the number of wounds studied was small, and more studies need to be done which include the use of growth factors and that compare this matrix to liquid application of the growth factor.

2. Rat dorsal subcutaneous implant study with PDGF. In this study, DOPA collagen cylinders with and without PDGF (20 ng/cylinder) were implanted into rats subcutaneously. Animals were sacrificed at 1,2,3,4,6, and 8 weeks after implantation and tissues removed and evaluated histologically. The results of this study showed that at 1 and 2 weeks, the PDGF-containing cylinders clearly had much greater infiltration of lymphocytes and fibroblasts as well as capillaries (blood vessels did not appear at all in control cylinders until later times). The differences in this study were very dramatic, and thus of some significance.

3. Human skin studies. The only human studies to date using iodine-collagen has been to apply the material to skin donor sites in burn patients. Patients reported a decrease in pain with the iodine-collagen dressing. However, no growth factor was included in this study, and there are only a few cases.

FGF IS RELEASED SLOWLY FROM DOPA-CROSSLINKED MATRICES

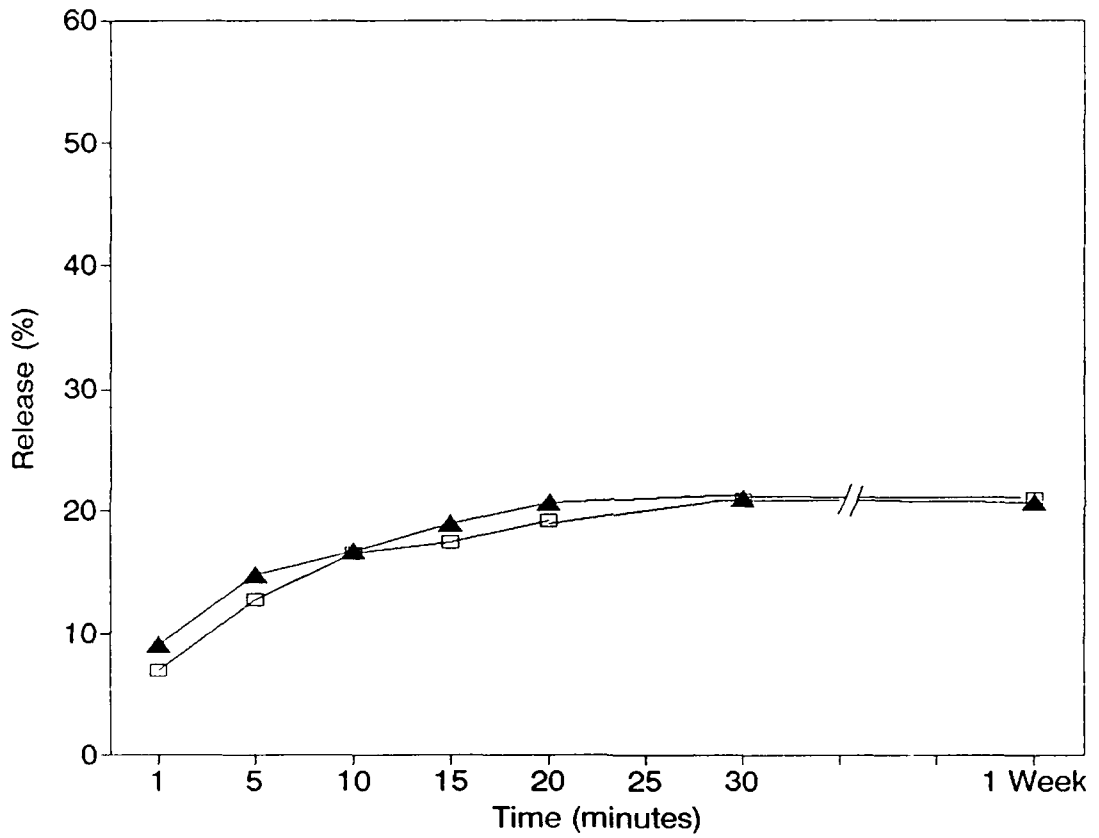

Figure 1. DOPA CROSSLINKED MATERIAL ACTS AS A SUSTAINED RELEASE MATRIX FOR FGF, AND THE RATE OF RELEASE IS NOT AFFECTED BY THE PRESENCE OF HEPARIN. 0.2 ml cylindrical discs of the material were made by allowing the DOPA/collagen mix to crosslink in sterile syringes, and then removing the cylinder and slicing the gel into 0.2 ml volume slices. In the experimental gels, FGF (SCIOS) and I-^{125}FGF were added to the DOPA/collagen mix before crosslinking.

▲ = dopa collagen disc with 50 ug heparin: □ = dopa collagen disc. The results show that about 20% of the material is released over the first 30 minutes, after which the release is markedly slower. Our data indicate that 80% of the incorporated FGF is retained in the gel for at least 1 week, and that this is not affected by the presence of heparin.

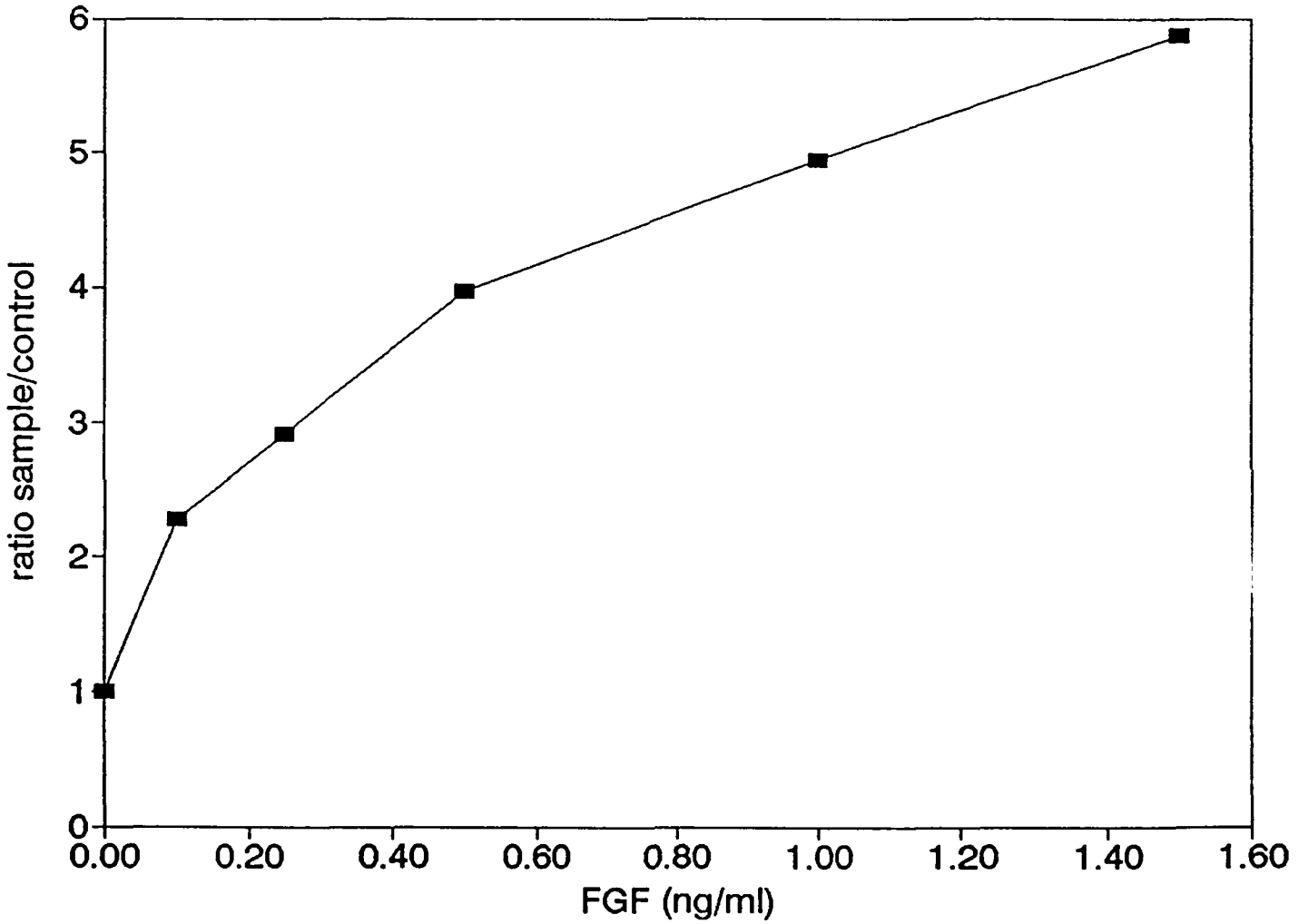

Figure 2. Response of human fibroblasts (HFF5) to FGF (Scios). Assay performed as described in the text. Data are expressed as ^{3}H-dT incorporation in test cultures divided by the ^{3}H-dT incorporation in control cultures (average 13,000 cpm/well). Numbers are the average of 4 cultures for each data point.

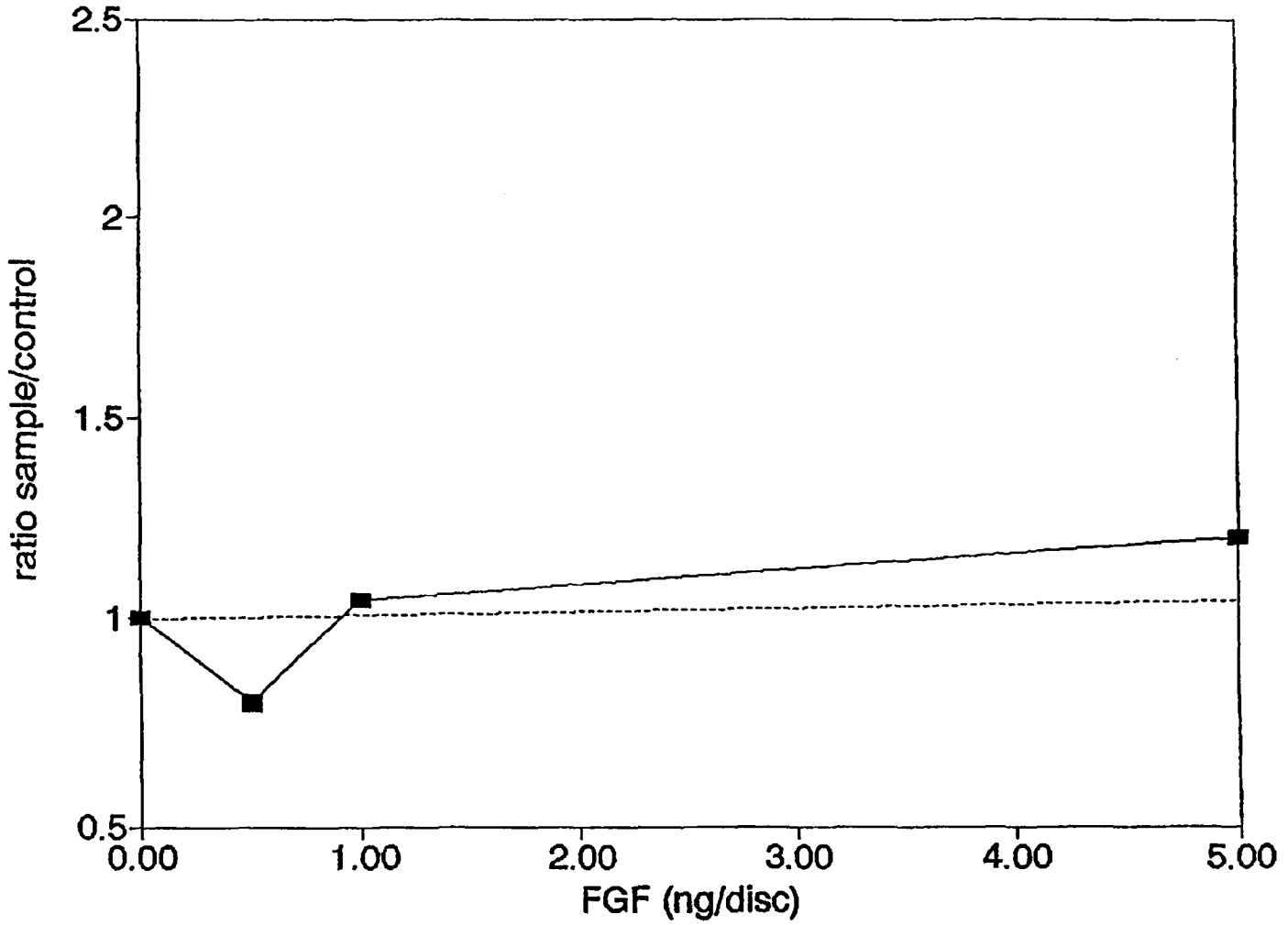

Figure 3. Response of HFF5 cells to FGF-containing iodine gelled collagen discs. Assay as described in the text. Data are expressed as ^{3}H-dT incorporation in test cultures divided by that in control cultures (average ^{3}H-dT incorporation was 13,000 cpm/well). Numbers are the average of at least 3 cultures for each data point.

Figure 4. Control rabbit ear, 2 weeks. Experiment performed as described in the text. Note that at the end of the cartilage, there is degradation of the matrix and chondrocyte death.

Figure 5. FGF-containing rabbit ear disc, 2 weeks. Experiment as described in the text. Note that at the cartilage end, the perichondrium is responding with dividing cells and that there is growth outward toward and underneath the implanted disc.